BIPHASIC BIOCATALYTIC TESTOSTERONE DEHYDROGENATION IN MICROFLUIDIC DROPLETS

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ABSTRACT

Two-phase biocatalysis is commonly used for the bioconversion of water-insoluble chemical and pharmaceutical compounds. In research laboratories, such processes often take place in flasks and are driven and limited by slow diffusive processes. In this work, we developed droplet-based microfluidic systems and used them to perform testosterone dehydrogenation. The use of microdroplets as reaction vessels offers the advantage of greatly improved diffusion rates due to significantly increased surface-to-volume ratio. We found that indeed reaction time was reduced from tens of minutes to tens of seconds without sacrificing conversion efficiency. This demonstrates the potential of droplet-based two-phase biocatalysis.

Keywords: two-phase biocatalysis, microdroplets

INTRODUCTION

In biocatalysis, enzymes or whole cells are used as catalysts for chemical transformations. Compared to chemical methods, biocatalysis offers several advantages such as high specificity, environmental friendliness, and reduced energy requirements due to reactions at room temperature and atmospheric pressure [Anthonsen, 2000; Faber, 2011].

Enzymes used as catalysts for chemical reactions are generally less stable in organic media compared to their natural aqueous environment, but chemical and pharmaceutical compounds are often insoluble in water [Klibanov, 2001]. The use of aqueous/organic two-phase systems provides a solution to this problem. Such two-phase systems are now commonly used in multiple biotransformations [Adebar, 2019]. The addition of a non-polar organic phase as a substrate reservoir and product sink can ensure high substrate loadings while avoiding enzyme inhibition or inactivation.

Figure 1 shows a simplified example of a traditional aqueous/organic two-phase system. In this configuration, biocatalysis is driven by diffusion. The substrate diffuses into the aqueous phase, where it is converted by the enzyme. In turn, the product diffuses back into the organic phase, where it is collected. Diffusion is often facilitated by shaking the flasks or stirring their contents. Mixing increases the contact area between the two phases, but at the same time mechanical stress associated with shaking or stirring can cause enzyme denaturation. The formation of emulsions during stirring is also a limitation of such systems, as phases are difficult to separate for product collection. Additionally, the amount or type of organic phase can also have an inhibitory effect on the reaction, as it can impede enzyme activity.

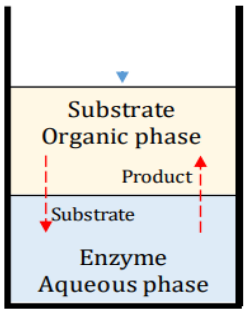

Figure 1: Aqueous/organic biphasic system

RESEARCH CONCEPT

In this work, we designed, fabricated, and tested microfluidic devices for droplet-based two-phase biocatalysis on-chip. The use of microfluidic droplets as

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miniaturized reactors offers the advantage of greatly improved diffusion rates due to the significantly increased surface-to-volume ratio [Žnidaršič-Plazl, 2014; Fernandes, 2010]. In turn, higher diffusion rates can increase the reaction conversion rate. Another advantage of microfluidic droplets is lack of mechanical stress associated with their formation and their relative ease of separation compared to emulsions created by active mixing in flasks. Microdroplet separation ensures efficient product extraction.

ENZYME-BASED BIOCATALYTIC REACTION

17β-Hydroxysteroid dehydrogenases (17β-HSDs) are a type of NAD(P)-dependent oxidoreductases. Their main function is to participate in the metabolism of sex hormones by controlling the last step in the formation of androgens and estrogens. Thus, they play an important role in reproduction [Oppermann, 1996; Benach, 1996]. Herein, 17β-hydroxysteroid dehydrogenase is used for testosterone dehydrogenation to androstenedione, as shown in Figure 2 below.

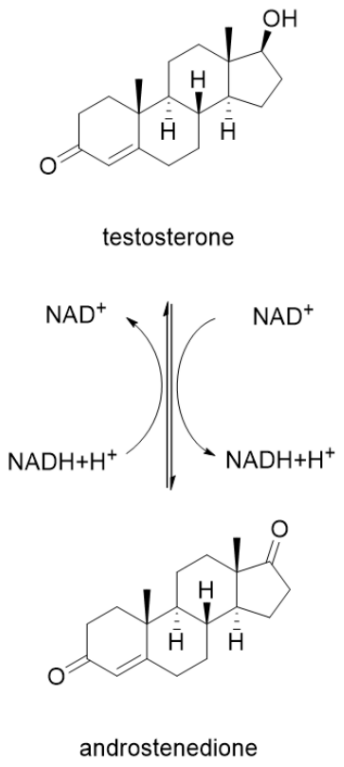

Figure 2: Conversion of testosterone to androstenedione catalyzed by 17β-hydroxysteroid dehydrogenase.

Androstenedione can be used as a precursor of hormones, such as male hormones and protein synthesis hormones, and can be used to synthesize pharmaceuticals such as spironolactone, hydrocortisone, prednisone oxide, and dexamethasone [Elks, 2014]. Testosterone is the main protein assimilation substance, and it is of great significance to health, affecting, among others, sexual desire, strength, and immune function [Mooradian, 1987].

MICROFLUIDIC DEVICE DESIGN AND FABRICATION

There are three typical microfluidic device architectures used to generate droplets: T-junctions, flow-focusing devices, and co-flowing devices [i Solvas, 2011]. The experiments described in this work were carried out in flow-focusing devices.

The droplets produced have an oil-in-water (O/W) structure. The continuous phase is a buffer containing the enzyme, and the discrete phase is an organic solvent containing the substrate testosterone. Figure 3 shows the geometry of the microfluidic device used in this study. To generate droplets, the continuous phase "squeezes" the fluid front of the dispersed phase from both sides of the intersection, and uses the "neck" structure of the channel downstream of the liquid front to form discrete droplets. The width of the continuous-phase microchannel, w_{con}, is 110 μm and the width of the discrete-phase microchannel, w_{di}, is 60 μm. The two phases converge in a narrow "neck" with a width of 50 μm and form droplets in a 400 μm channel.

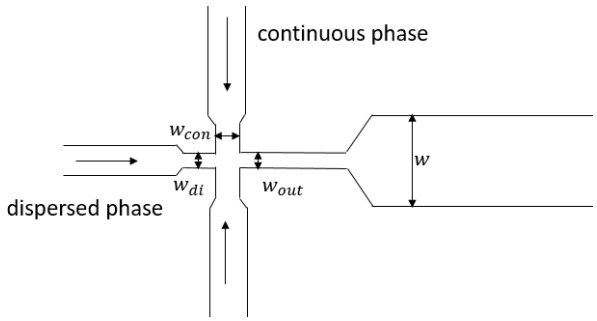

Figure 3: Geometry of microfluidic device used for droplet generation.

The microfluidic devices were fabricated in glass using femtosecond laser ablation. Glass was chosen due to its chemical inertness, which ensures no effects on the biocatalytic reaction. After laser ablation of microchannels, the glass wafer with a thickness of 700 nm was etched in hydrofluoric acid to remove surface defects, followed by cleaning in piranha solution (a mixture of sulfuric acid, water, and hydrogen peroxide), accompanied by an increase in surface hydrophilicity. To seal the microfluidic channels, the base wafer was thermally bonded to a cover glass wafer. The finished microfluidic device was therefore made exclusively out of transparent glass, which also allows the use of microscopy for process monitoring.

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REACTION DETAILS

The aqueous phase and organic phase solutions were prepared before the start of the experiment. 50 µl of a 10 µg/µl enzyme solution, 1000 µl of 20 mM nicotinamide adenine dinucleotide (NAD^+) and 3450 µl of buffer were mixed to form the aqueous phase. The buffer was composed of 20 mM Tris-HCl and 25 mM sodium chloride at pH 9. Methyl tertiary butyl ether (MTBE) was chosen as the organic solvent, in which the concentration of testosterone was 10 mM. In addition, 1% Tween 20 was added to the aqueous phase as a surfactant to reduce the surface tension and promote the formation and stabilization of the droplets.

CONTINUOUS BIOCATALYTIC REACTIONS IN MICRODROPLETS

Syringe pumps were used to push the organic and aqueous phase into microchannels, and experiments were carried out at two flow rates. In sample 1, the flow rate of the organic phase was 1.5 µl/min, and the flow rate of the aqueous phase was 4.5 µl/min. In sample 2, the flow rates of the organic and aqueous phases were 3 µl/min and 9 µl/min, respectively. At the outlet of the microfluidic system 250 µl of liquid was collected for analysis. Ethyl acetate was added to the collection container at a ratio of 1:1 (ethyl acetate to reaction solution) to terminate the reaction. In addition, a 6 cm-long PTFE tube with an inner diameter of 0.25 mm was installed at the outlet of the glass microfluidic chip to provide a longer residence time for the reaction. The residence time (i.e. reaction time) of the reaction solution in the system was approximately 40 s (sample 1) and 20 s (sample 2).

The droplets generated in the microchannel are shown in Figure 4. The average droplet diameter was approximately 80 µm.

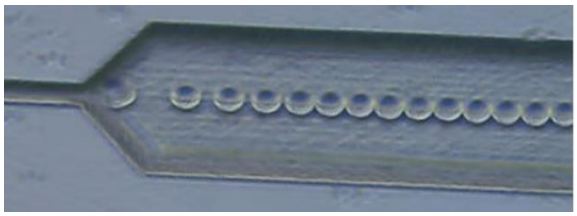

Figure 4: Microdroplets in the microchannel. The flow rate of the organic phase is 1.5 µl/min, and the flow rate of the water phase is 4.5 µl/min. Observed under a microscope. For scale, the width of the microchannel that contains droplets is 400 µm.

RESULTS AND DISCUSSION

High performance liquid chromatography (HPLC) was used for the detection of substrate and product in the solution. Figure 5 below shows the chromatogram of the reaction in sample 1. By calculating the ratio of the peak areas of the product and the substrate, the conversion of the substrate testosterone to the product androstenedione in the two reactions was determined as follows: sample 1: 91%, sample 2: 85%. The corresponding amount of the produced product were 0.5687 µmol and 0,53125 µmol, respectively.

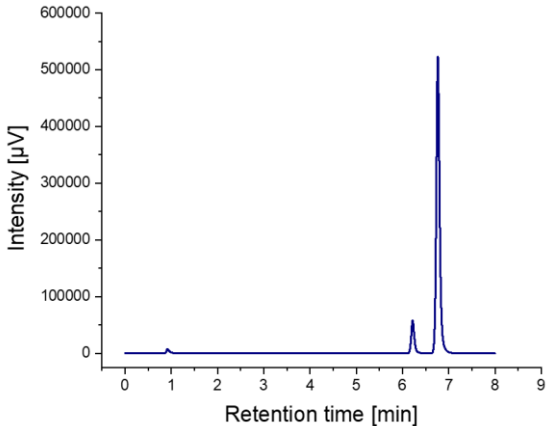

Figure 5: The chromatogram of the substrate and product in sample 1, where the abscissa represents the retention time and the ordinate represents the intensity. The retention times of testosterone and androstenedione are 6.2 min and 6.8 min respectively.

The same experiment was performed in a conventional batch reaction at the same substrate concentration of 10 mM and stirring at 900 rpm. The concentration of enzyme in the buffer was 50 µg/ml. As using large amounts of organic solvent can affect the activity of the enzyme, the ratio of the organic to aqueous phase used was 1:9. At a reaction time of 10 minutes, the conversion rate was 85 %. After 15 minutes, the conversion rate increased to 93 %.

CONCLUSIONS

The dehydrogenation reaction of testosterone catalyzed by 17β-HSD was realized in microdroplets and was compared to traditional batch experiments. We found that high conversion rates can be achieved in such miniaturized systems in significantly less time (tens of seconds) compared to the traditional setup (tens of minutes). This is partially due to much higher diffusion rates achieved in microsystems facilitated by the large droplet surface-to-volume areas. Therefore, such miniaturized reactors are a promising platform for the performance of two-phase biocatalysis.

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NOMENCLATURE

w	the width of the microchannel, μm

SUBSCRIPT

con	continuous-phase
di	dispersed-phase
out	outlet

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